(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/081542 A3

(51) International Patent Classification^7: G06K 9/00

(21) International Application Number: PCT/US01/12748

(22) International Filing Date: 20 April 2001 (20.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/556,497 21 April 2000 (21.04.2000) US

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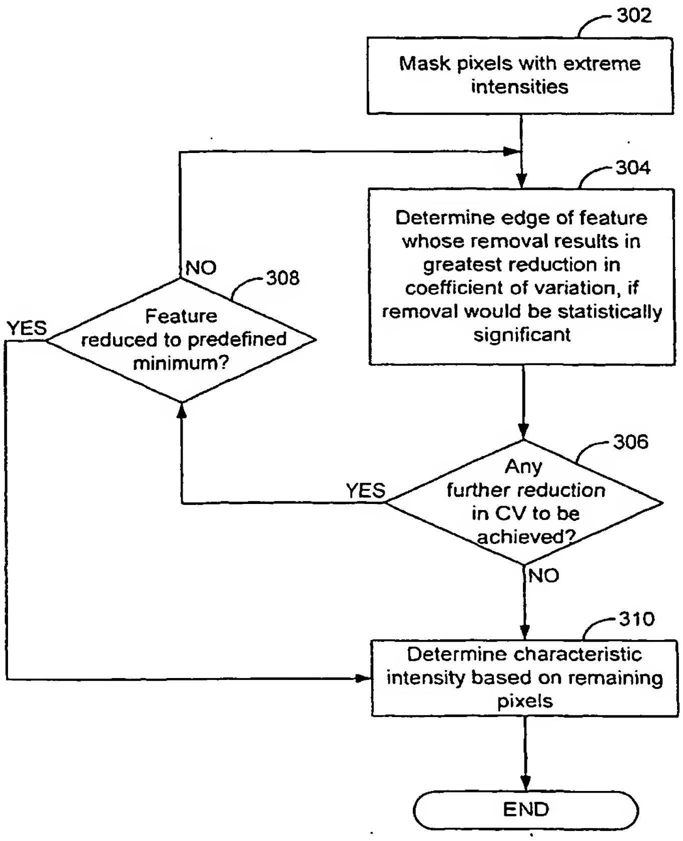
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,

[Continued on next page]

(54) Title: FEATURE EXTRACTION AND NORMALIZATION ALGORITHMS FOR HIGH-DENSITY OLIGONUCLEOTIDE GENE EXPRESSION ARRAY DATA


302
Mask pixels with extreme intensities
304
Determine edge of feature whose removal results in greatest reduction in coefficient of variation, if removal would be statistically significant
NO
308
YES
Feature reduced to predefined minimum?
306
YES
Any further reduction in CV to be achieved?
NO
310
Determine characteristic intensity based on remaining pixels
END

(57) Abstract: A characteristic intensity of a feature in image data generated by scanning a microarray probe is determined (304). A set of pixels of the image data that nominally represent the feature is identified. The pixels each have a value (such as an intensity value) associated therewith. For each of a plurality of subsets of the set of pixels, a variation statistic value is determined that corresponds to a variation in the values associated with the pixels of that subset (Fig.3).

NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

(88) Date of publication of the international search report:
7 March 2002

(48) Date of publication of this corrected version:
16 January 2003

(15) Information about Correction:
see PCT Gazette No. 03/2003 of 16 January 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

FEATURE EXTRACTION AND NORMALIZATION ALGORITHMS FOR HIGH-DENSITY OLIGONUCLEOTIDE GENE EXPRESSION ARRAY DATA

GOVERNMENT CONTRACT STATEMENT

At least some of the subject matter of this disclosure was developed while under contract with the National Science Foundation (grants DMS-9703918 and DBI-9904701).

TECHNICAL FIELD

The invention relates to the analysis of gene probe microarrays and, in particular, to the analysis of image data produced by such gene probe microarrays.

BACKGROUND

Monitoring gene expression using high-density microarrays is a technique in the study of cell functions and the associated biochemical pathways, candidate gene identification, cellular response to drug compounds, and classification of disease states. For example, see:

- Alon, U. et al. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues by oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA*, **96**, 6745-6750 (1999).
- Zhu, H. et al. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. Proc. Natl. Acad. Sci. USA 95, 14470-14475 (1998).
- Wodicka, L. et al. Genome-wide expression monitoring in Saccharomyces cerevisiae. Nature Biotechnology 15, 1359-1366 (1997).
- Eisen, M.B. et al. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci.USA 95, 14863-14868 (1998).
- Tamayo, P., et al. Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. Proc. Natl. Acad. Sci. USA 96, 2907-2912 (1999).
- Golub, T.R. et al. Molecular classification of cancer Class discovery and class prediction by gene expression monitoring. Science 286, 531-537 (1999).

It appears that recent research has largely focused on enhancing the microarray technology itself and the corresponding experimental protocols. For example, see

- Lockhart, D.J. et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* **14**, 1675-1680 (1996).
- Schena, M. et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467-470 (1995).
- Shalon, D. et al. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Research* **6**, 639-645 (1996).
- Mahadevappa, M. & Wodicka, L. A high-density probe array sample preparation method using 10- to 100-fold fewer cells. *Nature Biotechnology* **17**, 1134-1136 (1999).

Other research has focused on developing higher-level analysis methods such as clustering and classification. For example, see

- Chen, Y. et al. Ratio-based decisions and the quantitative analysis of cDNA microarray images. Journal of Biomedical Optics 2, 364-374 (1997).

Chen et al. detailed algorithms for image segmentation and confidence intervals for expression ratios for cDNA microarray data.

The fundamentals of oligonucleotide expression array technology are described, for example, in the Lockhart paper cited above and are well-known in the art. The oligonucleotide expression array technology is broadly discussed here to provide a frame of reference for discussion of the invention. In particular, genes are represented on a probe array by some number of sequences of a particular length that uniquely identify the genes and that ostensibly have optimal hybridization characteristics. Each oligonucleotide (probe) is synthesized in a small cell that contains a large number (typically between 10^6 and 10^7) of copies of a given probe.

A mismatch (MM) oligonucleotide is designed to correspond to a perfect match (PM) oligonucleotide pulled from a gene sequence. In an MM oligonucleotide, typically the center base position of the oligonucleotide has been mutated. The MM probes give some estimate of the random hybridization and cross hybridization signals.

To use an oligonucleotide array, RNA samples are prepared and fluorescently labeled according to a particular protocol (e.g., the protocol set forth by Lockhart et al. in the article cited above), and then the labeled RNA sample is hybridized to the corresponding probes on the array. The array then goes through an automated staining/washing process (e.g., using an Affymetrix fluidics station), and the array is scanned using a confocal laser. The scanner generates an image of the array by exciting each cell with its laser, detects the resulting photon emissions from the fluorescently labeled RNA that has hybridized to the probes in the cell, and then converts the detected photon emissions into a raw intensity value for each cell. "Features" (comprised of groups of cells) are "extracted" based on the images, and characteristic feature intensities are computed from the raw cell intensities. It can be determined from the features' "characteristic intensity" whether a particular gene is present in the array, and the quantity at which the gene is present.

Conventional feature extraction is now discussed in greater detail. For example, as discussed by Wodicka et al.(1997), the raw oligonucleotide array image has recognizable patterns at each corner that allows the determination of the positions of the corners of the array. The number of features in each row and column is known. Once the corners are determined, the positions of each feature in the array are computed.

As can be seen from Fig. 1A, the boundary pixels of a feature are typically distorted by blurring (i.e. their levels are "pulled" towards the level of a neighboring feature) and do not faithfully represent the true intensity of the feature. Therefore, the boundary pixels are conventionally removed before the characteristic feature intensity is computed. That is, the intensities of the boundary pixels of a feature are not considered in determining a characteristic intensity value for the feature. In most cases, after removing the boundary pixels from a feature, the feature is represented by a 6 x 6 block of pixels that remain.

Then, the characteristic intensity for the feature is determined, for example, by computing an average intensity of the remaining pixels. It can be seen from Fig. 1A that determining the median of the remaining 6 x 6 pixels often results in determining the median value from a more variable region than, say, the most homogenous block of pixels (e.g., a 4 x 4 pixel block) within the 6 x 6 pixel block. This can result in a downward bias from the "true" characteristic feature intensity.

Furthermore, Fig. 1B illustrates how a misalignment of the basic grid can result in a failure to extract the central part of the true feature.

What is desired is a feature extraction method that more robustly and reliably extracts the "useful" portion of a true feature for determining characteristic feature intensity.

Furthermore, it is well known that the comparison of gene expression results across experiments is enhanced when the results of the experiments are normalized to a single scale. Normalizing multiple probe arrays to allow direct array-to-array comparisons has presented a great challenge. Conventional normalization methods include 1) linear normalization and nonlinear regression, and 2) methods using housekeeping genes or staggered spike-in controls.

With linear normalization, it is assumed that the intensities between two or more arrays are related as a straight line with a zero y-intercept. Its use leads to multiplication by a scaling factor (slope of the line) to make the mean of the "experiment" chip the same as that of the baseline chip. A description of this technique applied to Affymetrix probe arrays is given by Alon et al. (1999). For example, see page 6746, lines 2-4 which states that

> "To compensate for possible varaitions between arrays, the intensity of each EST on an array was divided by the mean intensities of all ESTs on that array and multiplied by a nominal average intensity of 50."

Ignoring the slight differences of the number of retained probe pairs per gene (due to outlier probe removal), the essential effect of these operations is equivalent to the multiplication of each probe pair difference by a constant scaling factor.

Chen et al. (1998) describe an application of the linear normalization technique to cDNA spotted arrays, where one intensity channel is normalized against another on the same array. For example, on page 371, formulae (12) & (13) represent a linear scaling operation across the whole array. (The Chen paper actually used a more complicated procedure where the scaling is applied iteratively in connection with the fitting of the density for the ratios.)Although the linear normalization technique is simple and robust, this method has the drawback that it does not account well for nonlinear relations. For example, Fig. 2 illustrates a situation where the slope in the low intensity region (of the scatter plot of PM/MM differences between two arrays) is substantially different from the slope in the high intensity region. In fact, a 10%-50% difference in slope values between regions is quite common. A non-linear regression technique (e.g., generalized cross-validation or GCVSS as described in Wahba, G. *Spline Methods for Observational Data*. CBMS-NSF regional conference series in applied mathematics. Philadelphia: SIAM (1990)) may be employed, but even non-linear regression can be inadequate if the expression profiles of the various arrays vary greatly from each other.

The drawbacks of the conventional normalization methods can be seen with reference to Fig. 2. Specifically the PM/MM difference from two murine Affymetrix Mu6500SubA probe array experiments are plotted in Fig. 2. The line 202 is the line generated by the LR normalization method, the curve 204 is generated by the GCVSS method, and the lines 206 and 208 are generated by applying the LR method to low and high differences, respectively (the low/high cutoff was determined empirically from the GCVSS line 204 matches the line 206 at the low end and the 208 and 202 lines at the high end, although the data between the two experiments is not really linearly related.

It has also been suggested (e.g., see Ermolaeva et al. 1998) that normalization between arrays can be based on a set of "housekeeping" genes. Unfortunately, many of the genes conventionally used as housekeeping genes (e.g., β actin, glyceraldehyde-3-phosphate dehydrogenase, transferrin receptor, signal transducer and activator of transcription 1, among others) have ranges of differential expression similar to other genes whose differential expression patterns are deemed biologically relevant to the system under study. In accordance with one known method, control cRNAs for bacterial and phage genes (e.g., BioB, BioC, BioD, and cre) are consistently added to hybridization mixtures at known concentrations. However, these controls are often prepared in bulk and completely independently of the sample being profiled, and so, the normalization relation between the controls on different arrays typically does not reflect the true normalization relation for the biologically relevant genes of interest.

What is desired is a normalization method that more accurately reflects the true normalization relation.

SUMMARY

In accordance with one aspect of the invention, a method is provided to determine a characteristic intensity of a feature in image data generated by scanning a microarray probe. A set of pixels of the image data that nominally represent the feature is identified. The pixels each have an value (such as an intensity value) associated therewith. For each of a plurality of subsets of the set of pixels, a variation statistic value is determined that corresponds to a variation in the values associated with the pixels of that subset. One of the subsets of pixels is chosen based on the determined variation statistic values.

In accordance with a further aspect of the invention, a method is provided to relate a first expression array of probes to a second expression array of probes. A subset of the probe for the arrays is determined based on a comparison of the ordering of the subset of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes. A relationship of the second expression array to the first expression array is determined based on the subset of probes of the second expression array to the corresponding probes of the first array.

BRIEF DESCRIPTION OF FIGURES

Fig. 1A illustrates how boundary pixels “blur” a feature, while Fig. 1B illustrates how grid mismatch contributes to inadequate feature extraction.

Fig. 2 is a scatter plot with the PM-MM slope superimposed thereon, illustrating the drawbacks of conventional normalization techniques.

Fig. 3 is a flow chart illustrating an embodiment of an adaptive pixel selection (APS) method in accordance with the invention.

Fig. 4 is a flow chart illustrating an embodiment of an invariant difference selection (IDS) method, particularly with the invention and useful in normalizing results between various microarrays.

Fig. 5 is a scatter plot of PM-MM differences with IDS/GCVSS normalization curve superimposed thereon, the differences for normalization were selected in accordance with an embodiment of the invention.

DETAILED DESCRIPTION

What is now described, broadly speaking, is a method for analyzing raw data generated by gene expression microarrays, including oligonucleotide expression microarrays. Such an array may include the microarray known as the GeneChip® probe array provided by Affymetrix of Santa Clara, California. Steps of feature extraction and normalization are particularly described.

Feature Extraction

Now, an adaptive pixel selection (APS) method for feature extraction is described with reference to Fig. 3. The APS method is carried out on a set of pixels at least nominally on pixels preliminarily identified as being part of a feature. At step 302, the pixels with extreme intensities are masked. (For example, pixels more than three standard deviations from the mean pixel value within a feature, including the boundary pixels of the feature, are removed.) At step 304, it is determined which edge of the feature, if its pixels are removed, would result in the greatest reduction in the coefficient of variation (CV) of the remaining pixels. Preferably the pixels of the edge are removed only if the reduction in CV is statistically significant.

At step 306, it is determined if any further reductions in CV can be achieved by removing another edge. If so, it is determined at step 308 if the feature is already reduced to a predetermined minimum number of pixels. If no further reduction in CV can be achieved or if the feature is already reduced to the predetermined number of pixels, then at step 310 the characteristic intensity of the feature is determined from the remaining pixels (e.g., by taking a mean of the raw intensity values for the remaining pixels). Otherwise, steps 304, 306 and 308 are repeated.

In some embodiments, the pixel selection process just described is constrained by forcing adjacent subregions selected by the process (corresponding to adjacent features) to be separated by at least two pixels. It is thought that forcing such a separation tends to select the most homogenous group of pixels whose mean value is used to represent the intensity for the given feature.

In another embodiment, rather than "shrinking" the set of pixels under consideration, the set of pixels under consideration is instead "grown" from a seed pixel (or set of pixels) until a subset of pixels corresponding to the feature is achieved that has an appropriately minimal CV. In this case, "appropriately minimal" may be the subset of pixels that has the absolute minimum CV. For example, in accordance with one such "growing" embodiment, the central 2x2 pixel block of the nominal feature pixels is selected as the seed region. Then, either a row or column is added to the already-selected region. Typically, the row or column is chosen that, when added, results in the smallest increase in variability of pixel intensity of the selected region. The growing step is iteratively repeated until a predetermined size is achieved (e.g., 4x4 pixels or 5x5 pixels). In addition, in some embodiments, the adding step is constrained such that the resulting enhanced region does not deviate too drastically from a particular shape (typically square).

In an experiment, the APS "shrinking" method was compared to the median method, by examining twelve replicate oligonucleotide arrays. Replicate data were generated by hybridizing the same cRNA hybridization cocktail onto six high-density Affymetrix Hu6800 probe arrays and six "A" probe arrays from the low-density Affymetrix Hu6800 four-chip set. The twelve arrays were normalized using the IDS/GCVSS normalization method described below. It is reasonable to expect, after normalization, the intensities for any given feature across the twelve replicates to be roughly equal, since the same sample was hybridized onto each array. For each feature across the twelve replicate arrays, the feature-intensity standard deviations (SD) were computed after using the median and the APS methods to determine the feature intensities.

A "good" feature extraction algorithm would lead to a small SD among the replicates. For these arrays, 74% of the APS feature-intensity SDs were smaller than the corresponding median feature-intensity SDs and, in these cases, the average ratio between the median-computed SD to the APS-computed SD was 2.10. On the other hand, the average ratio between the APS-computed SDs and the median-computed SDs was only 1.27, when the APS-computed SD was larger than the median-computed SD. Thus the APS algorithm leads to a 40% reduction in the intensity standard deviation across replicate arrays, when compared to the median algorithm.

Given that these feature intensity calculations form the basis for all future analyses, any method that significantly reduces the measurement error will serve to increase the sensitivity and specificity of the experiment.

Normalization

Now, normalization is described with reference to Figs. 4 and 5,. The normalization is carried out by a so-called "invariant difference selection" method

(IDS). Broadly speaking, the IDS method chooses a subset of some features of an array for normalization based on differences between some characteristic of those features. For example, the feature intensities or the PM/MM intensity differences may serve as the basis for fitting a normalization relation.

Using the PM/MM differences as an illustrative example, a set of probes are said to be invariant if the ordering of these probes according to the PM/MM differences in the experiment array, is the same as that in the baseline array. Intuitively, if a gene is truly differentially expressed, then the PM/MM differences for this gene are more likely to have different ranks relative to the other probes, and hence they are not likely to be included in a large invariant set. In some embodiments, the maximal invariant set is computed using a dynamic programming algorithm. However the resulting set is typically too small to form a reliable normalization curve. In another embodiment, the IDS method finds an approximately invariant set of differences that is not necessary maximal, but is large enough for reliable fitting of the normalization relation.

Turning now to Fig.4, in accordance with one embodiment, various subsets of the PM-MM difference values are input to a difference calculation. That is, at step 402, the following expressions are evaluated for the input differences:

$$R_i = \left[L(B_i + E_i) + H(2N - B_i - E_i)\right] \Big/ 2N$$

$$D_i = 2\left|B_i - E_i\right| \Big/ (B_i + E_i)$$

where L and H are the rank difference thresholds for the low and high ends of the difference intensity range, B_i and E_i are the ranks for the i^{th} difference of the baseline and experiment arrays, and N is the total number of differences that were ordered in the current iteration of the method. R_i defines the threshold for difference intensity i by linearly interpolating the threshold between a low difference intensity threshold, given by L, and a high difference intensity threshold, given by H.

The interpolation addresses the property that there are many more points at low intensities than at high intensities. While a more strict threshold policy can practically be enforced at the low end, it is more practical to relax the threshold constraint at the high end such that enough points are obtained in this range to reliably establish the normalization relation. D_i is the rank difference test statistic used to determine if the i^{th} difference should be included in the invariant set, for the current iteration of the algorithm. The i^{th} difference is considered approximately invariant if $D_i < R_i$.

At step 404, the difference value and rank difference is stored for the particular input set. The selection process then repeats at steps 402 and 404, taking the current set of approximately invariant differences as input, until all differences meet the threshold criteria.

At step 406, the approximately invariant set of differences has been selected and the normalization curve is constructed (e.g., by applying the GCVSS technique described in Wahba 1990 to the invariant set). An example of such a normalization curve is shown in Fig. 5. Fig. 5 shows the same data presented in Fig. 2. The black points represent the differences determined by the IDS technique, and the line is the GCVSS fit of the invariant differences between the baseline and the experiment arrays.

The efficacy of the just-described IDS/GVCSS is now discussed. In comparing the IDS/GCVSS normalization technique to the linear regression (LR) and standalone GCVSS methods, criteria that could be established to test the superiority of one normalization method over another are: 1) the method minimizes the PM/MM intensity difference variances across a series of replicate arrays, and 2) the method preserves expression ratios in simulated data. The first criterion addresses the desire that genes known to have identical expression levels have observed levels as close to identical as possible. The second criterion addresses the desire that criterion 1 not be achieved at the expense of destroying the very biological variation the technology aims to detect.

Table 1 presents the results of comparing the IDS/GCVSS method against the GCVSS method and the LR method, carried out on the same set of twelve replicate probe arrays discussed above. In particular, Table 1 shows results of comparing average difference intensities across a series of twelve replicate probe arrays using no normalization (UN), linear normalization (LR), smoothing spline normalization (GCVSS) and the invariant difference selection/smoothing spline (IDS/GCVSS) methods described in the text. The first number in each value pair of Table 1 represents the percentage of standard deviations, computed across the twelve replicates for each of the genes, that were larger when the normalization technique listed in the leftmost column was compared against the normalization technique listed along the top row. For instance, in cell (LR, IDS) the value pair (0.65, 1.5) indicates that 65% of the difference standard deviations were larger when LR normalization was used compared to the difference standard deviations when the IDS method was used, and of those that were larger, the median LR/IDS ratio of standard deviations was 1.5, i.e., for 65% of the genes, half of those had an LR standard deviation that was more than 1.5 times larger than the corresponding IDS standard deviations.

The results shown in Table 1 indicate that the IDS/GCVSS method is reasonably similar to the standalone GVCSS method, and both are better at making the average of the PM/MM differences for a particular gene, i.e., the average difference intensities, across these replicate arrays more consistent than doing nothing at all or than using the LR method.

> Relation	UN	LR	GCVSS	IDS
UN	N/A	(0.96,1.2)	(0.85,1.7)	(0.74,1.8)
LR	(0.04,1.0)	N/A	(0.76,1.4)	(0.65,1.5)
GCVSS	(0.15,1.3)	(0.24,1.2)	N/A	(0.46,1.4)
IDS	(0.26,1.2)	(0.35,1.3)	(0.54,1.5)	N/A

Table 1

Results from two sets of simulated expression-ratio data are presented in Table 2. In the first set, 300 genes that were consistently detected as present across the 6 low-density replicate probe arrays and 600 from the high-density replicate probe arrays were randomly selected; 6 sets containing 50 genes each for the low-density arrays and 100 genes each for the high-density arrays were then generated by randomly selecting, without replacement, from the sets of 300 and 600 randomly selected genes. The PM/MM differences comprising each of the genes in each of the sets were then multiplied by 2.0, 0.5, 4.0, 0.25, 6.0, and 0.17, respectively, to simulate fold changes between samples. The twelve original replicate probe arrays as well as the twelve modified replicate probe arrays were then normalized using the normalization techniques listed in Table 1.

> Relation	LR.1/LR.2	GCVSS.1/GCVSS.2	IDS.1/IDS.2
LR.1/LR.2	N/A	(0.08, 1.2)/(0.15, 1.3)	(0.94, 9.3)/(0.61, 2.1)
GCVSS.1/GCVSS.2	(0.92, 3.2)/(0.85, 3.2)	N/A	(0.99, 16.5)/(0.95, 3.9)
IDS.1/IDS.2	(0.06, 1.4)/(0.39, 1.4)	(0.01, 1.1)/(0.05, 1.3)	N/A

Table 2

The same procedure was applied to the second set, except that 320 genes from the low density arrays and 640 genes from the high-density arrays were randomly selected, and then 16 sets of 20 genes/40 genes each were formed and the corresponding difference intensities were multiplied by 2.0, 0.5, 2.5, 0.4, 3.0, 0.33, 3.75, 0.27, 4.0, 0.25, 5.0, 0.20, 6.0, 0.17, 7.0 and 0.14, respectively, yielding a very diverse differential expression pattern in which as many as 18% of the genes on the arrays were forced to be differentially expressed. The standard deviations of the differences between the true fold change and the observed fold change after normalization were computed for each of the modified genes.

In table 2, the first value in each value pair represents the percentage of standard deviations that were larger when the normalization technique listed in the leftmost column was compared against the normalization technique listed along the top row. For instance, the first value pair (0.94, 9.3) in cell (LR.1/LR.2, IDS.1/IDS.2) indicates that for the first set of data 94 percent of the LR.1 computed standard deviations were larger than the IDS.1 computed standard deviations, and that of those that were larger, the median LR.1/IDS.1 ratio of standard deviations was 9.3, i.e., half of the time, the LR.1 standard deviation is more than 9.3 times larger than the IDS.1 standard deviation.

Table 2, shows that the GCVSS technique makes the average difference intensities more consistent by destroying the very biological variation the technology aims to detect. That is, Table 2 shows how the GCVSS technique is too sensitive to the relatively small number of genes that change. On the other hand, the IDS/GCVSS technique continues to perform better than the other techniques, not only by minimizing the average difference intensity variation across replicates, but also by minimizing the deviation from the true fold change values in the simulated data.

As mentioned above in the introduction to this “Normalization” section, feature intensities may be used for normalization, too. Thus, finally, in another embodiment, normalization is performed on feature intensities rather than PM-MM differences. The method is the same as in the IDS/GCVSS method described above, except that the scatter plot of corresponding differences between the two arrays is replaced by the scatter plot of the corresponding feature intensity values between the two arrays.

Conclusion

Processes for feature extraction and normalization have been described. Because many important decisions as to whether a gene should be pursued as a candidate for a particular biological system under study are directly based on determined expression ratios as well as on the determined differential expression calls, processes that provide for more accurate estimates of these derived statistics can be valuable to users of oligonucleotide array technology.

It should be noted that the image processing techniques may have broader application beyond oligonucleotide arrays or even to microarrays, and claim to such broader application is not intended to be foreclosed by this disclosure.

WHAT IS CLAIMED IS:

1. A method of determining a characteristic intensity of a feature in image data generated by scanning a microarray probe, comprising:

identifying a set of pixels of the image data that nominally represent the feature, wherein the pixels each have an intensity value associated therewith;

for each of a plurality of subsets of the set of pixels, determining a variation statistic value that corresponds to a variation in the intensity values associated with the pixels of that subset; and

choosing one of the subsets of pixels based on the determined variation statistic values.

2. The method of claim 1, and further comprising:

determining the characteristic intensity of the feature based on the intensity values associated with the pixels of the chosen subset.

3. The method of claim 2, wherein the subsets of the set of pixels are determined in an iterative fashion starting from a "seed" set of pixels and forming subsequent subsets by adding additional pixels.

4. The method of claim 3, wherein each subsequent subset is chosen at each iteration from predetermined additional subsets to have the smallest determined variation statistic value of the predetermined additional subsets.

5. The method of claim 4, wherein the predetermined additional subsets all have substantially a predetermined shape.

6. A method of determining a characteristic value for a feature in image data generated by scanning a microarray probe, comprising:

identifying a set of pixels of the image data that nominally represent the feature, wherein the pixels each have a value associated therewith;

for each of a plurality of subsets of the set of pixels, determining a variation statistic value that corresponds to a variation in the values associated with the pixels of that subset;

choosing one of the subsets of pixels based on the determined variation statistic values.

7. The method of claim 6, and further comprising:

determining the characteristic value of the feature based on the intensity values associated with the pixels of the chosen subset.

8. The method of claim 7, wherein the subsets of the set of pixels are determined in an iterative fashion starting from a "seed" set of pixels and forming subsequent subsets by adding additional pixels.

9. The method of claim 8, wherein each subsequent subset is chosen at each iteration from predetermined additional subsets to have the smallest determined variation statistic value of the predetermined additional subsets.

10. The method of claim 9, wherein the predetermined additional subsets all have substantially a predetermined shape.

11. A method of relating a first expression array of probes to a second expression array of probes, comprising:

determining a subset of the probes for the arrays based on a comparison of the ordering of the subset of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes; and

determining a relationship of the second expression array to the first expression array based on the subset of probes of the second expression array to the corresponding probes of the first array.

12. The method of claim 11, wherein the step of determining a subset of probes includes:

selecting the determined subset of probes from a plurality of subsets of the probes.

13. The method of claim 12, wherein the selecting step comprises:

comparing the ordering of a first of the plurality of subsets of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes;

if the comparison does not meet a particular criterion, repeating the comparing step with a second of the plurality of subsets of the probes of the second array, wherein the second subset is a subset of the first subset;

using the subset of the probes for which the comparison meets the particular criterion to determine the relationship of the second expression array to the first expression array.

14. The method of claim 13, wherein the particular criterion is a threshold.

15. The method of claim 14, wherein the threshold is not identical for all the probes.

16. The method of claim 13, wherein the step of using the probes to determine the relationship includes applying a nonlinear regression technique to the subset of the probes.

17. The method of claim 16, wherein the step of applying a nonlinear regression technique includes applying a generalized cross-validation step to the subset of the probes of the second array and the corresponding probes of the first array.

18. The method of claim 14, wherein:

the comparison D_i and threshold R_i are determined by the equations:

$$R_i = {[L(B_i + E_i) + H(2N - B_i - E_i)]} \Big/ {2N}$$

$$D_i = {2|B_i - E_i|} \Big/ {(B_i + E_i)}$$

where L and H are rank difference thresholds for the low and high ends of the range of characteristic values, B_i and E_i are the ranks for the i^{th} characteristic value of the baseline and experiment arrays, and N is the total number of characteristic values that were ordered in the current iteration of the method.

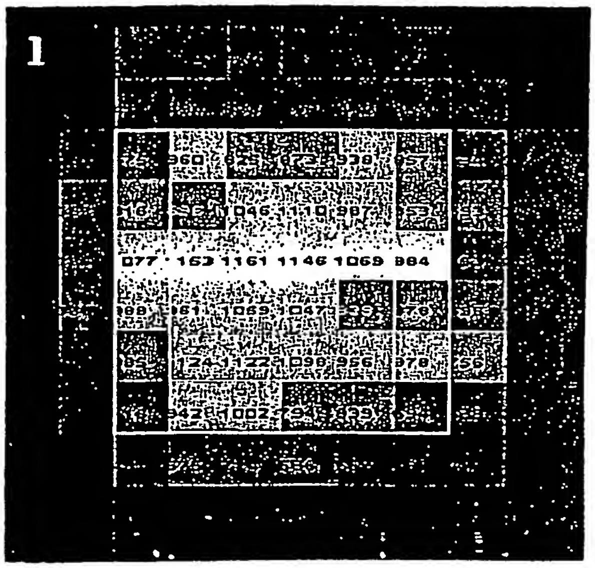


FIG. 1A. PRIOR ART

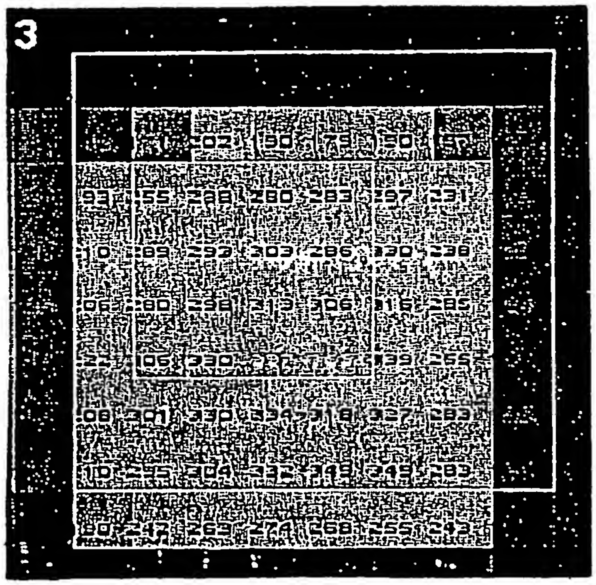


FIG. 1B. PRIOR ART

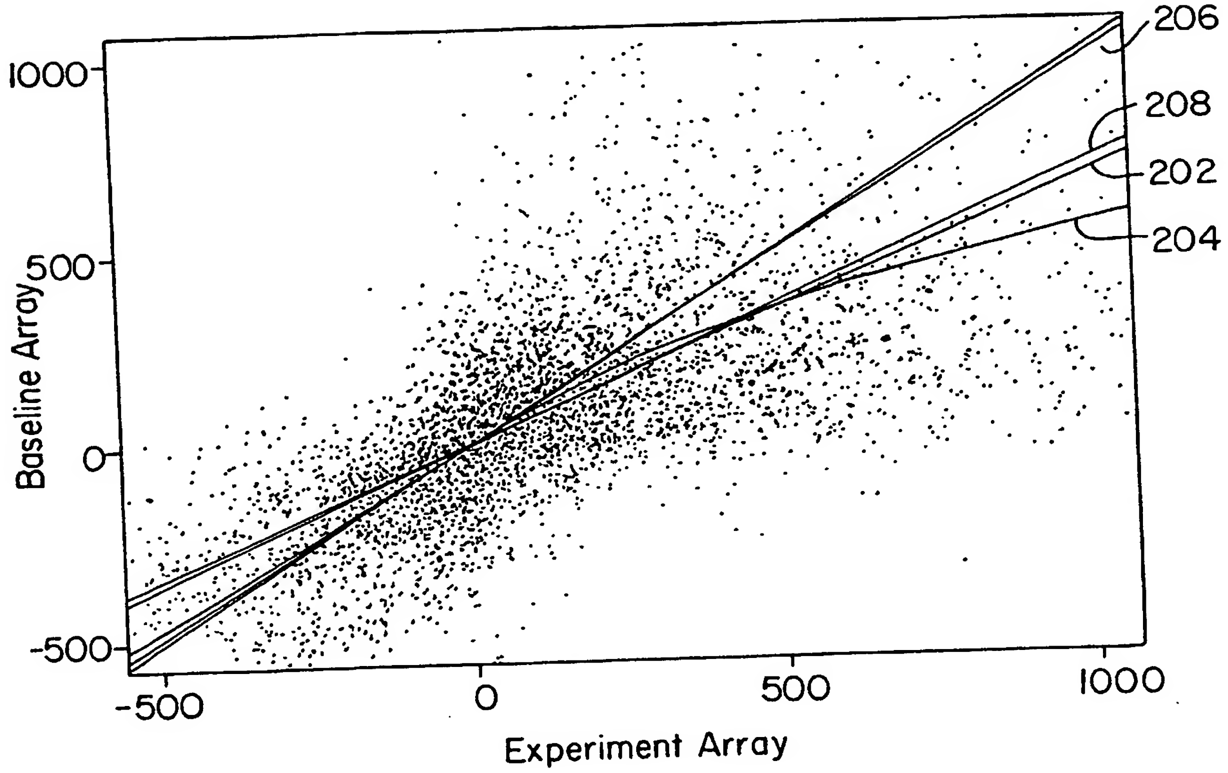

206
208
202
204
1000
500
0
-500
Baseline Array
-500
0
500
1000
Experiment Array

FIG. 2.

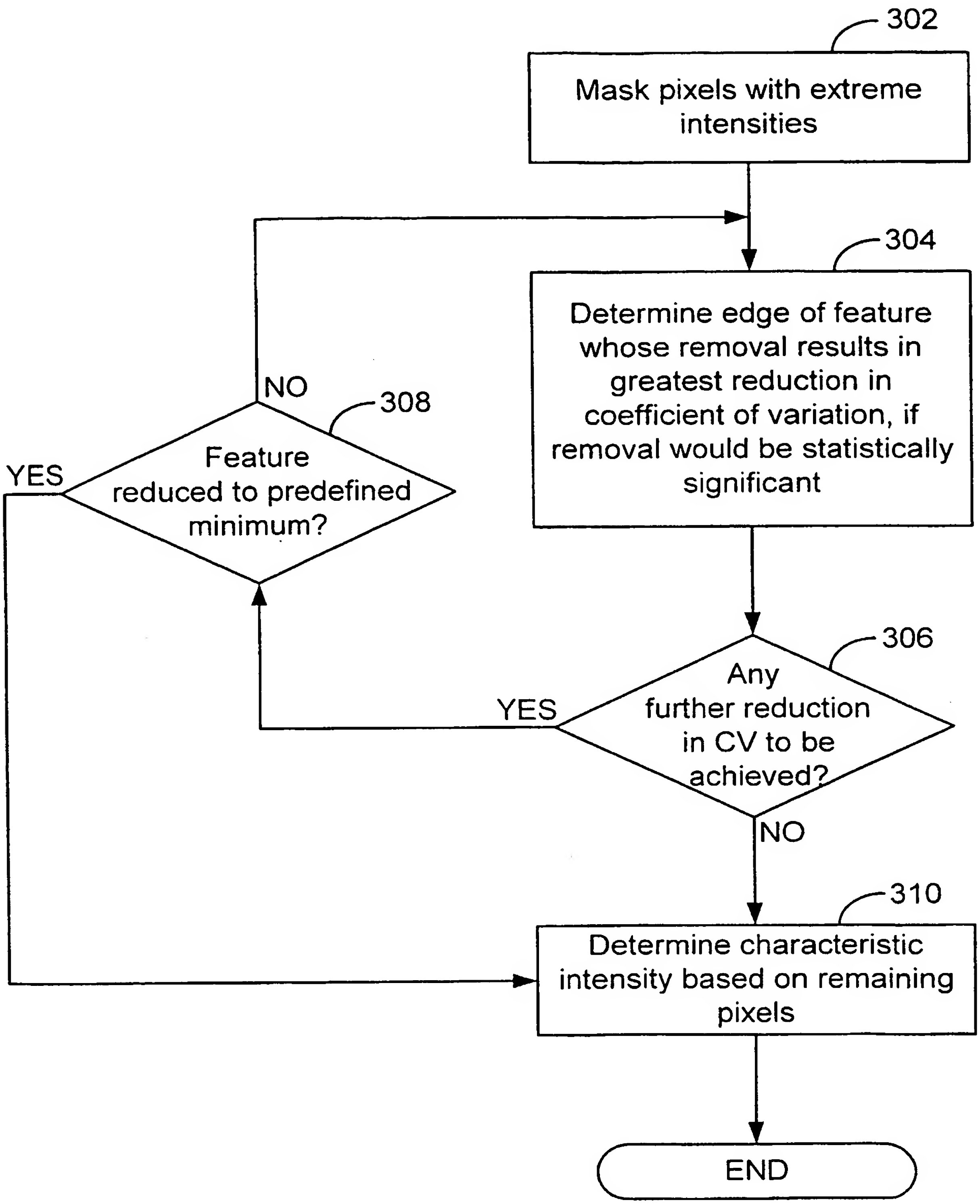

302
Mask pixels with extreme intensities
304
Determine edge of feature whose removal results in greatest reduction in coefficient of variation, if removal would be statistically significant
306
Any further reduction in CV to be achieved?
YES
NO
308
Feature reduced to predefined minimum?
NO
YES
310
Determine characteristic intensity based on remaining pixels
END

FIG. 3.

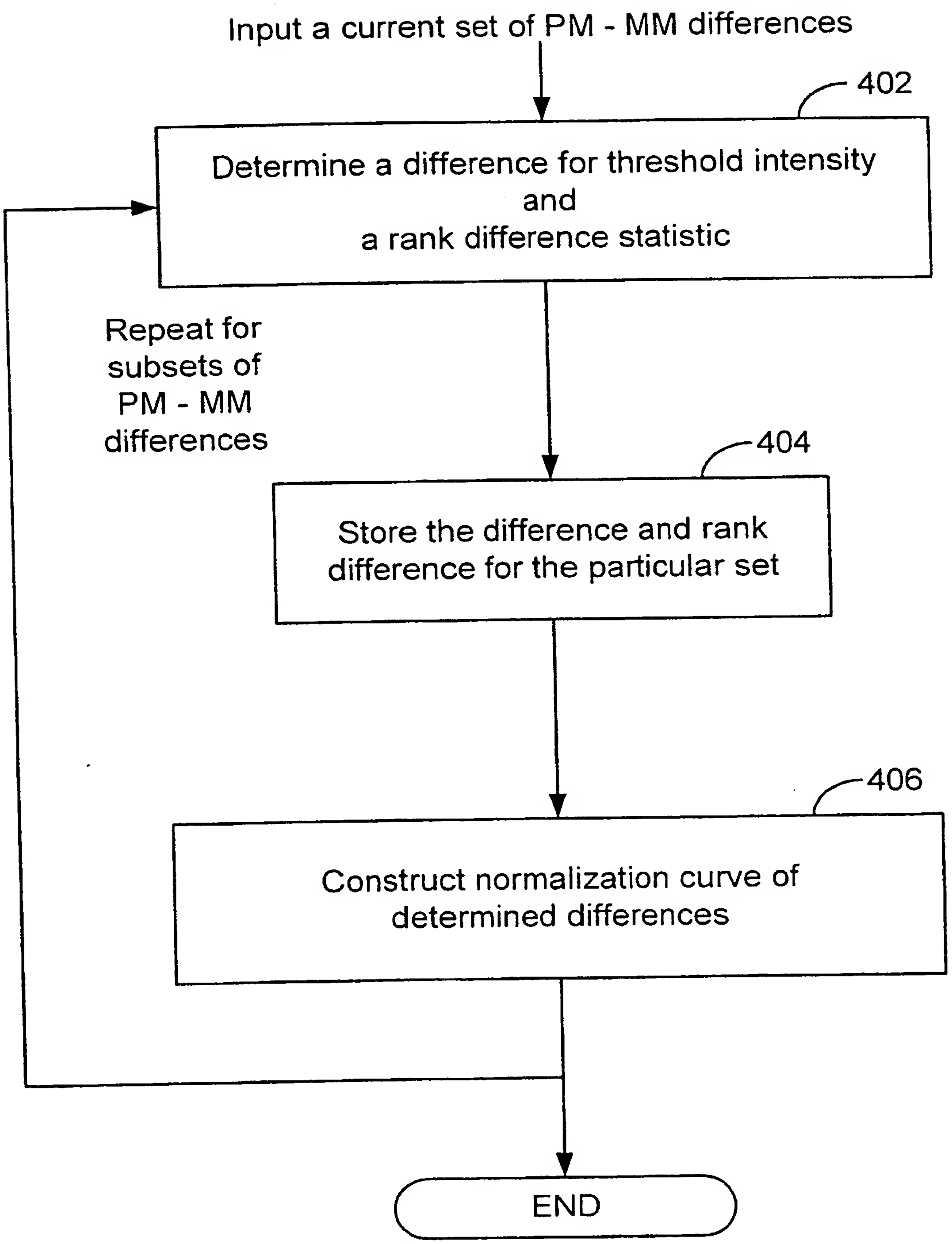

Input a current set of PM - MM differences
402
Determine a difference for threshold intensity
and
a rank difference statistic
Repeat for
subsets of
PM - MM
differences
404
Store the difference and rank
difference for the particular set
406
Construct normalization curve of
determined differences
END

FIG. 4.

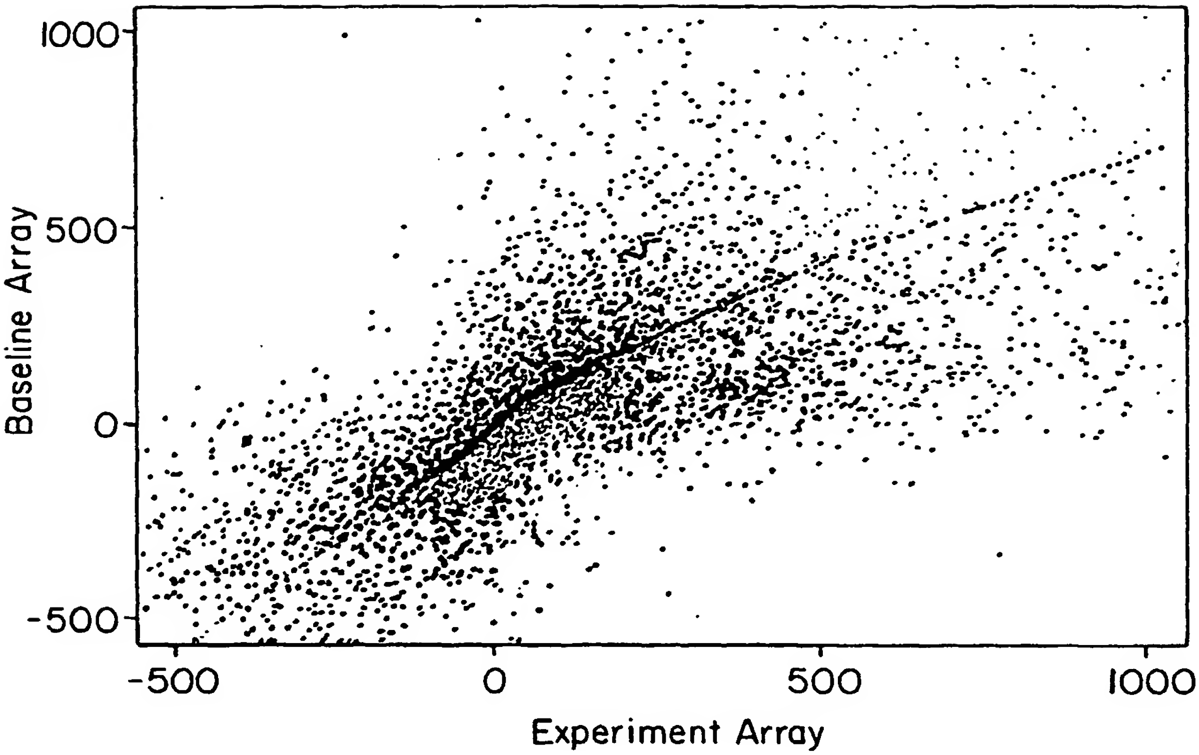

1000
500
0
-500
Baseline Array
-500
0
500
1000
Experiment Array

FIG. 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12748

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G06K 9/00
US CL : 382/128,133
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 382/128,133

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,790,692 A (PRICE et al) 04 August 1998.	1-18

□ Further documents are listed in the continuation of Box C. □ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 01 JUNE 2001	Date of mailing of the international search report 30 OCT 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MAHMOOD B. CHOOBIN Telephone No. (703) 306-5787

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/81542 A3

(51) International Patent Classification⁷: G06K 9/00

(21) International Application Number: PCT/US01/12748

(22) International Filing Date: 20 April 2001 (20.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/556.497 21 April 2000 (21.04.2000) US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 5th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors; and
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(74) Agent: DONNELLY, Darren, E.; McCutchen Doyle Brown & Enersen, LLP, Three Embarcadero Center, San Francisco, CA 94111 (US).

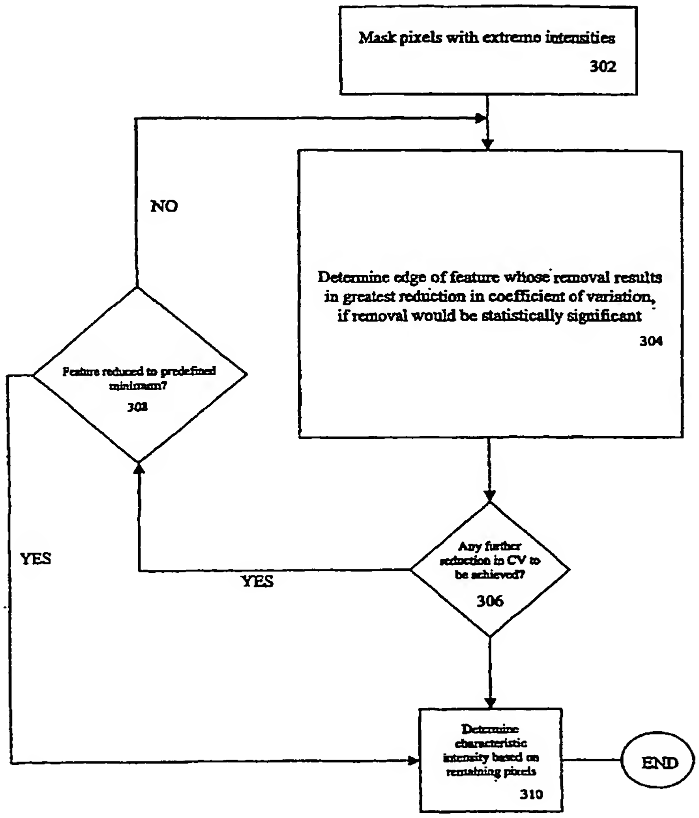
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: FEATURE EXTRACTION AND NORMALIZATION ALGORITHMS FOR HIGH-DENSITY OLIGONUCLEOTIDE GENE EXPRESSION ARRAY DATA

(57) Abstract: A characteristic intensity of a feature in image data generated by scanning a microarray probe is determined (304). A set of pixels of the image data that nominally represent the feature is identified. The pixels each have a value (such as an intensity value) associated therewith. For each of a plurality of subsets of the set of pixels, a variation statistic value is determined that corresponds to a variation in the values associated with the pixels of that subset (Fig.3).


Mask pixels with extremo intensities
302
Determine edge of feature whose removal results in greatest reduction in coefficient of variation, if removal would be statistically significant
304
NO
Feature reduced to predefined minimum?
308
YES
YES
Any further reduction in CV to be achieved?
306
Determine characteristic intensity based on remaining pixels
310
END

IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

(88) Date of publication of the international search report: 7 March 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12748

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"&" document member of the same patent family

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/81542 A2

(51) International Patent Classification⁷: C12N

(21) International Application Number: PCT/US01/12748

(22) International Filing Date: 20 April 2001 (20.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/556,497 21 April 2000 (21.04.2000) US

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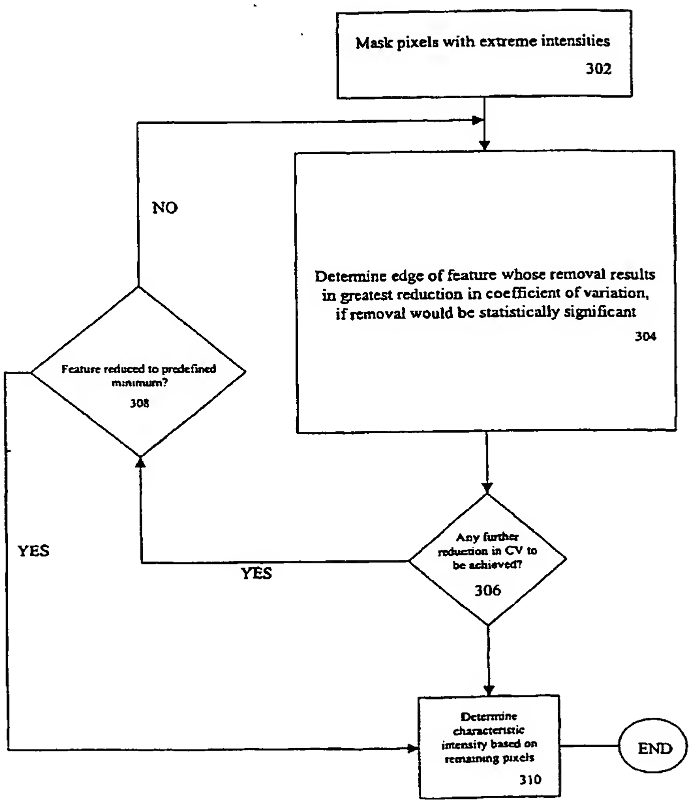
[Continued on next page]

(54) Title: FEATURE EXTRACTION AND NORMALIZATION ALGORITHMS FOR HIGH-DENSITY OLIGONUCLEOTIDE GENE EXPRESSION ARRAY DATA


Mask pixels with extreme intensities
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304
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308
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YES
Any further reduction in CV to be achieved?
306
Determine characteristic intensity based on remaining pixels
310
END

(57) Abstract: A characteristic intensity of a feature in image data generated by scanning a microarray probe is determined. A set of pixels of the image data that nominally represent the feature is identified. The pixels each have a value (such as an intensity value) associated therewith. For each of a plurality of subsets of the set of pixels, a variation statistic value is determined that corresponds to a variation in the values associated with the pixels of that subset. One of the subsets of pixels is chosen based on the determined variation statistic values. A method is also described to relate a first expression array of probes to a second expression array of probes. A subset of the probes for the arrays is determined based on a comparison of the ordering of the subset of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes. A relationship of the second expression array to the first expression array is determined based on the subset of probes of the second expression array to the corresponding probes of the first array.

IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

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FEATURE EXTRACTION AND NORMALIZATION ALGORITHMS FOR HIGH-DENSITY OLIGONUCLEOTIDE GENE EXPRESSION ARRAY DATA

GOVERNMENT CONTRACT STATEMENT

At least some of the subject matter of this disclosure was developed while under contract with the National Science Foundation (grants DMS-9703918 and DBI-9904701).

TECHNICAL FIELD

The invention relates to the analysis of gene probe microarrays and, in particular, to the analysis of image data produced by such gene probe microarrays.

BACKGROUND

Monitoring gene expression using high-density microarrays is a technique in the study of cell functions and the associated biochemical pathways, candidate gene identification, cellular response to drug compounds, and classification of disease states. For example, see:

- Alon, U. et al. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues by oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA*, 96, 6745-6750 (1999).
- Zhu, H. et al. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. Proc. Natl. Acad. Sci. USA 95, 14470-14475 (1998).
- Wodicka, L. et al. Genome-wide expression monitoring in Saccharomyces cerevisiae. Nature Biotechnology 15, 1359-1366 (1997).
- Eisen, M.B. et al. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci.USA 95, 14863-14868 (1998).
- Tamayo, P., et al. Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. Proc. Natl. Acad. Sci. USA 96, 2907-2912 (1999).
- Golub, T.R. et al. Molecular classification of cancer Class discovery and class prediction by gene expression monitoring. Science 286, 531-537 (1999).

It appears that recent research has largely focused on enhancing the microarray technology itself and the corresponding experimental protocols. For example, see

- Lockhart, D.J. et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* **14**, 1675-1680 (1996).
- Schena, M. et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467-470 (1995).
- Shalon, D. et al. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Research* **6**, 639-645 (1996).
- Mahadevappa, M. & Wodicka, L. A high-density probe array sample preparation method using 10- to 100-fold fewer cells. *Nature Biotechnology* **17**, 1134-1136 (1999).

Other research has focused on developing higher-level analysis methods such as clustering and classification. For example, see

- Chen, Y. et al. Ratio-based decisions and the quantitative analysis of cDNA microarray images. Journal of Biomedical Optics 2, 364-374 (1997).

Chen et al. detailed algorithms for image segmentation and confidence intervals for expression ratios for cDNA microarray data.

The fundamentals of oligonucleotide expression array technology are described, for example, in the Lockhart paper cited above and are well-known in the art. The oligonucleotide expression array technology is broadly discussed here to provide a frame of reference for discussion of the invention. In particular, genes are represented on a probe array by some number of sequences of a particular length that uniquely identify the genes and that ostensibly have optimal hybridization characteristics. Each oligonucleotide (probe) is synthesized in a small cell that contains a large number (typically between 10^6 and 10^7) of copies of a given probe.

A mismatch (MM) oligonucleotide is designed to correspond to a perfect match (PM) oligonucleotide pulled from a gene sequence. In an MM oligonucleotide, typically the center base position of the oligonucleotide has been mutated. The MM probes give some estimate of the random hybridization and cross hybridization signals.

To use an oligonucleotide array, RNA samples are prepared and fluorescently labeled according to a particular protocol (e.g., the protocol set forth by Lockhart et al. in the article cited above), and then the labeled RNA sample is hybridized to the corresponding probes on the array. The array then goes through an automated staining/washing process (e.g., using an Affymetrix fluidics station), and the array is scanned using a confocal laser. The scanner generates an image of the array by exciting each cell with its laser, detects the resulting photon emissions from the fluorescently labeled RNA that has hybridized to the probes in the cell, and then converts the detected photon emissions into a raw intensity value for each cell. "Features" (comprised of groups of cells) are "extracted" based on the images, and characteristic feature intensities are computed from the raw cell intensities. It can be determined from the features' "characteristic intensity" whether a particular gene is present in the array, and the quantity at which the gene is present.

Conventional feature extraction is now discussed in greater detail. For example, as discussed by Wodicka et al.(1997), the raw oligonucleotide array image has recognizable patterns at each corner that allows the determination of the positions of the corners of the array. The number of features in each row and column is known. Once the corners are determined, the positions of each feature in the array are computed.

As can be seen from Fig. 1A, the boundary pixels of a feature are typically distorted by blurring (i.e. their levels are "pulled" towards the level of a neighboring feature) and do not faithfully represent the true intensity of the feature. Therefore, the boundary pixels are conventionally removed before the characteristic feature intensity is computed. That is, the intensities of the boundary pixels of a feature are not considered in determining a characteristic intensity value for the feature. In most cases, after removing the boundary pixels from a feature, the feature is represented by a 6 x 6 block of pixels that remain.

Then, the characteristic intensity for the feature is determined, for example, by computing an average intensity of the remaining pixels. It can be seen from Fig. 1A that determining the median of the remaining 6 x 6 pixels often results in determining the median value from a more variable region than, say, the most homogenous block of pixels (e.g., a 4 x 4 pixel block) within the 6 x 6 pixel block. This can result in a downward bias from the "true" characteristic feature intensity.

Furthermore, Fig. 1B illustrates how a misalignment of the basic grid can result in a failure to extract the central part of the true feature.

What is desired is a feature extraction method that more robustly and reliably extracts the "useful" portion of a true feature for determining characteristic feature intensity.

Furthermore, it is well known that the comparison of gene expression results across experiments is enhanced when the results of the experiments are normalized to a single scale. Normalizing multiple probe arrays to allow direct array-to-array comparisons has presented a great challenge. Conventional normalization methods include 1) linear normalization and nonlinear regression, and 2) methods using housekeeping genes or staggered spike-in controls.

With linear normalization, it is assumed that the intensities between two or more arrays are related as a straight line with a zero y-intercept. Its use leads to multiplication by a scaling factor (slope of the line) to make the mean of the "experiment" chip the same as that of the baseline chip. A description of this technique applied to Affymetrix probe arrays is given by Alon et al. (1999). For example, see page 6746, lines 2-4 which states that

> "To compensate for possible varaitions between arrays, the intensity of each EST on an array was divided by the mean intensities of all ESTs on that array and multiplied by a nominal average intensity of 50."

Ignoring the slight differences of the number of retained probe pairs per gene (due to outlier probe removal), the essential effect of these operations is equivalent to the multiplication of each probe pair difference by a constant scaling factor.

Chen et al. (1998) describe an application of the linear normalization technique to cDNA spotted arrays, where one intensity channel is normalized against another on the same array. For example, on page 371, formulae (12) & (13) represent a linear scaling operation across the whole array. (The Chen paper actually used a more complicated procedure where the scaling is applied iteratively in connection with the fitting of the density for the ratios.)Although the linear normalization technique is simple and robust, this method has the drawback that it does not account well for nonlinear relations. For example, Fig. 2 illustrates a situation where the slope in the low intensity region (of the scatter plot of PM/MM differences between two arrays) is substantially different from the slope in the high intensity region. In fact, a 10%-50% difference in slope values between regions is quite common. A non-linear regression technique (e.g., generalized cross-validation or GCVSS as described in Wahba, G. *Spline Methods for Observational Data*. CBMS-NSF regional conference series in applied mathematics. Philadelphia: SIAM (1990)) may be employed, but even non-linear regression can be inadequate if the expression profiles of the various arrays vary greatly from each other.

The drawbacks of the conventional normalization methods can be seen with reference to Fig. 2. Specifically the PM/MM difference from two murine Affymetrix Mu6500SubA probe array experiments are plotted in Fig. 2. The line 202 is the line generated by the LR normalization method, the curve 204 is generated by the GCVSS method, and the lines 206 and 208 are generated by applying the LR method to low and high differences, respectively (the low/high cutoff was determined empirically from the GCVSS line 204 matches the line 206 at the low end and the 208 and 202 lines at the high end, although the data between the two experiments is not really linearly related.

It has also been suggested (e.g., see Ermolaeva et al. 1998) that normalization between arrays can be based on a set of "housekeeping" genes. Unfortunately, many of the genes conventionally used as housekeeping genes (e.g., β actin, glyceraldehyde-3-phosphate dehydrogenase, transferrin receptor, signal transducer and activator of transcription 1, among others) have ranges of differential expression similar to other genes whose differential expression patterns are deemed biologically relevant to the system under study. In accordance with one known method, control cRNAs for bacterial and phage genes (e.g., BioB, BioC, BioD, and cre) are consistently added to hybridization mixtures at known concentrations. However, these controls are often prepared in bulk and completely independently of the sample being profiled, and so, the normalization relation between the controls on different arrays typically does not reflect the true normalization relation for the biologically relevant genes of interest.

What is desired is a normalization method that more accurately reflects the true normalization relation.

SUMMARY

In accordance with one aspect of the invention, a method is provided to determine a characteristic intensity of a feature in image data generated by scanning a microarray probe. A set of pixels of the image data that nominally represent the feature is identified. The pixels each have an value (such as an intensity value) associated therewith. For each of a plurality of subsets of the set of pixels, a variation statistic value is determined that corresponds to a variation in the values associated with the pixels of that subset. One of the subsets of pixels is chosen based on the determined variation statistic values.

In accordance with a further aspect of the invention, a method is provided to relate a first expression array of probes to a second expression array of probes. A subset of the probe for the arrays is determined based on a comparison of the ordering of the subset of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes. A relationship of the second expression array to the first expression array is determined based on the subset of probes of the second expression array to the corresponding probes of the first array.

BRIEF DESCRIPTION OF FIGURES

Fig. 1A illustrates how boundary pixels "blur" a feature, while Fig. 1B illustrates how grid mismatch contributes to inadequate feature extraction.

Fig. 2 is a scatter plot with the PM-MM slope superimposed thereon, illustrating the drawbacks of conventional normalization techniques.

Fig. 3 is a flow chart illustrating an embodiment of an adaptive pixel selection (APS) method in accordance with the invention.

Fig. 4 is a flow chart illustrating an embodiment of an invariant difference selection (IDS) method, particularly with the invention and useful in normalizing results between various microarrays.

Fig. 5 is a scatter plot of PM-MM differences with IDS/GCVSS normalization curve superimposed thereon, the differences for normalization were selected in accordance with an embodiment of the invention.

DETAILED DESCRIPTION

What is now described, broadly speaking, is a method for analyzing raw data generated by gene expression microarrays, including oligonucleotide expression microarrays. Such an array may include the microarray known as the GeneChip® probe array provided by Affymetrix of Santa Clara, California. Steps of feature extraction and normalization are particularly described.

Feature Extraction

Now, an adaptive pixel selection (APS) method for feature extraction is described with reference to Fig. 3. The APS method is carried out on a set of pixels at least nominally on pixels preliminarily identified as being part of a feature. At step 302, the pixels with extreme intensities are masked. (For example, pixels more than three standard deviations from the mean pixel value within a feature, including the boundary pixels of the feature, are removed.) At step 304, it is determined which edge of the feature, if its pixels are removed, would result in the greatest reduction in the coefficient of variation (CV) of the remaining pixels. Preferably the pixels of the edge are removed only if the reduction in CV is statistically significant.

At step 306, it is determined if any further reductions in CV can be achieved by removing another edge. If so, it is determined at step 308 if the feature is already reduced to a predetermined minimum number of pixels. If no further reduction in CV can be achieved or if the feature is already reduced to the predetermined number of pixels, then at step 310 the characteristic intensity of the feature is determined from the remaining pixels (e.g., by taking a mean of the raw intensity values for the remaining pixels). Otherwise, steps 304, 306 and 308 are repeated.

In some embodiments, the pixel selection process just described is constrained by forcing adjacent subregions selected by the process (corresponding to adjacent features) to be separated by at least two pixels. It is thought that forcing such a separation tends to select the most homogenous group of pixels whose mean value is used to represent the intensity for the given feature.

In another embodiment, rather than "shrinking" the set of pixels under consideration, the set of pixels under consideration is instead "grown" from a seed pixel (or set of pixels) until a subset of pixels corresponding to the feature is achieved that has an appropriately minimal CV. In this case, "appropriately minimal" may be the subset of pixels that has the absolute minimum CV. For example, in accordance with one such "growing" embodiment, the central 2x2 pixel block of the nominal feature pixels is selected as the seed region. Then, either a row or column is added to the already-selected region. Typically, the row or column is chosen that, when added, results in the smallest increase in variability of pixel intensity of the selected region. The growing step is iteratively repeated until a predetermined size is achieved (e.g., 4x4 pixels or 5x5 pixels). In addition, in some embodiments, the adding step is constrained such that the resulting enhanced region does not deviate too drastically from a particular shape (typically square).

In an experiment, the APS "shrinking" method was compared to the median method, by examining twelve replicate oligonucleotide arrays. Replicate data were generated by hybridizing the same cRNA hybridization cocktail onto six high-density Affymetrix Hu6800 probe arrays and six "A" probe arrays from the low-density Affymetrix Hu6800 four-chip set. The twelve arrays were normalized using the IDS/GCVSS normalization method described below. It is reasonable to expect, after normalization, the intensities for any given feature across the twelve replicates to be roughly equal, since the same sample was hybridized onto each array. For each feature across the twelve replicate arrays, the feature-intensity standard deviations (SD) were computed after using the median and the APS methods to determine the feature intensities.

A "good" feature extraction algorithm would lead to a small SD among the replicates. For these arrays, 74% of the APS feature-intensity SDs were smaller than the corresponding median feature-intensity SDs and, in these cases, the average ratio between the median-computed SD to the APS-computed SD was 2.10. On the other hand, the average ratio between the APS-computed SDs and the median-computed SDs was only 1.27, when the APS-computed SD was larger than the median-computed SD. Thus the APS algorithm leads to a 40% reduction in the intensity standard deviation across replicate arrays, when compared to the median algorithm.

Given that these feature intensity calculations form the basis for all future analyses, any method that significantly reduces the measurement error will serve to increase the sensitivity and specificity of the experiment.

Normalization

Now, normalization is described with reference to Figs. 4 and 5,. The normalization is carried out by a so-called "invariant difference selection" method

(IDS). Broadly speaking, the IDS method chooses a subset of some features of an array for normalization based on differences between some characteristic of those features. For example, the feature intensities or the PM/MM intensity differences may serve as the basis for fitting a normalization relation.

Using the PM/MM differences as an illustrative example, a set of probes are said to be invariant if the ordering of these probes according to the PM/MM differences in the experiment array, is the same as that in the baseline array. Intuitively, if a gene is truly differentially expressed, then the PM/MM differences for this gene are more likely to have different ranks relative to the other probes, and hence they are not likely to be included in a large invariant set. In some embodiments, the maximal invariant set is computed using a dynamic programming algorithm. However the resulting set is typically too small to form a reliable normalization curve. In another embodiment, the IDS method finds an approximately invariant set of differences that is not necessary maximal, but is large enough for reliable fitting of the normalization relation.

Turning now to Fig.4, in accordance with one embodiment, various subsets of the PM-MM difference values are input to a difference calculation. That is, at step 402, the following expressions are evaluated for the input differences:

$$R_i = {[L(B_i + E_i) + H(2N - B_i - E_i)]}\Big/{2N}$$

$$D_i = {2|B_i - E_i|}\Big/{(B_i + E_i)}$$

where L and H are the rank difference thresholds for the low and high ends of the difference intensity range, B_i and E_i are the ranks for the i^{th} difference of the baseline and experiment arrays, and N is the total number of differences that were ordered in the current iteration of the method. R_i defines the threshold for difference intensity i by linearly interpolating the threshold between a low difference intensity threshold, given by L, and a high difference intensity threshold, given by H.

The interpolation addresses the property that there are many more points at low intensities than at high intensities. While a more strict threshold policy can practically be enforced at the low end, it is more practical to relax the threshold constraint at the high end such that enough points are obtained in this range to reliably establish the normalization relation. D_i is the rank difference test statistic used to determine if the i^{th} difference should be included in the invariant set, for the current iteration of the algorithm. The i^{th} difference is considered approximately invariant if $D_i < R_i$.

At step 404, the difference value and rank difference is stored for the particular input set. The selection process then repeats at steps 402 and 404, taking the current set of approximately invariant differences as input, until all differences meet the threshold criteria.

At step 406, the approximately invariant set of differences has been selected and the normalization curve is constructed (e.g., by applying the GCVSS technique described in Wahba 1990 to the invariant set). An example of such a normalization curve is shown in Fig. 5. Fig. 5 shows the same data presented in Fig. 2. The black points represent the differences determined by the IDS technique, and the line is the GCVSS fit of the invariant differences between the baseline and the experiment arrays.

The efficacy of the just-described IDS/GVCSS is now discussed. In comparing the IDS/GCVSS normalization technique to the linear regression (LR) and standalone GCVSS methods, criteria that could be established to test the superiority of one normalization method over another are: 1) the method minimizes the PM/MM intensity difference variances across a series of replicate arrays, and 2) the method preserves expression ratios in simulated data. The first criterion addresses the desire that genes known to have identical expression levels have observed levels as close to identical as possible. The second criterion addresses the desire that criterion 1 not be achieved at the expense of destroying the very biological variation the technology aims to detect.

Table 1 presents the results of comparing the IDS/GCVSS method against the GCVSS method and the LR method, carried out on the same set of twelve replicate probe arrays discussed above. In particular, Table 1 shows results of comparing average difference intensities across a series of twelve replicate probe arrays using no normalization (UN), linear normalization (LR), smoothing spline normalization (GCVSS) and the invariant difference selection/smoothing spline (IDS/GCVSS) methods described in the text. The first number in each value pair of Table 1 represents the percentage of standard deviations, computed across the twelve replicates for each of the genes, that were larger when the normalization technique listed in the leftmost column was compared against the normalization technique listed along the top row. For instance, in cell (LR, IDS) the value pair (0.65, 1.5) indicates that 65% of the difference standard deviations were larger when LR normalization was used compared to the difference standard deviations when the IDS method was used, and of those that were larger, the median LR/IDS ratio of standard deviations was 1.5, i.e., for 65% of the genes, half of those had an LR standard deviation that was more than 1.5 times larger than the corresponding IDS standard deviations.

The results shown in Table 1 indicate that the IDS/GCVSS method is reasonably similar to the standalone GVCSS method, and both are better at making the average of the PM/MM differences for a particular gene, i.e., the average difference intensities, across these replicate arrays more consistent than doing nothing at all or than using the LR method.

> Relation	UN	LR	GCVSS	IDS
UN	N/A	(0.96,1.2)	(0.85,1.7)	(0.74,1.8)
LR	(0.04,1.0)	N/A	(0.76,1.4)	(0.65,1.5)
GCVSS	(0.15,1.3)	(0.24,1.2)	N/A	(0.46,1.4)
IDS	(0.26,1.2)	(0.35,1.3)	(0.54,1.5)	N/A

Table 1

Results from two sets of simulated expression-ratio data are presented in Table 2. In the first set, 300 genes that were consistently detected as present across the 6 low-density replicate probe arrays and 600 from the high-density replicate probe arrays were randomly selected; 6 sets containing 50 genes each for the low-density arrays and 100 genes each for the high-density arrays were then generated by randomly selecting, without replacement, from the sets of 300 and 600 randomly selected genes. The PM/MM differences comprising each of the genes in each of the sets were then multiplied by 2.0, 0.5, 4.0, 0.25, 6.0, and 0.17, respectively, to simulate fold changes between samples. The twelve original replicate probe arrays as well as the twelve modified replicate probe arrays were then normalized using the normalization techniques listed in Table 1.

> Relation	LR.1/LR.2	GCVSS.1/GCVSS.2	IDS.1/IDS.2
LR.1/LR.2	N/A	(0.08, 1.2)/(0.15, 1.3)	(0.94, 9.3)/(0.61, 2.1)
GCVSS.1/GCVSS.2	(0.92, 3.2)/(0.85, 3.2)	N/A	(0.99, 16.5)/(0.95, 3.9)
IDS.1/IDS.2	(0.06, 1.4)/(0.39, 1.4)	(0.01, 1.1)/(0.05, 1.3)	N/A

Table 2

The same procedure was applied to the second set, except that 320 genes from the low density arrays and 640 genes from the high-density arrays were randomly selected, and then 16 sets of 20 genes/40 genes each were formed and the corresponding difference intensities were multiplied by 2.0, 0.5, 2.5, 0.4, 3.0, 0.33, 3.75, 0.27, 4.0, 0.25, 5.0, 0.20, 6.0, 0.17, 7.0 and 0.14, respectively, yielding a very diverse differential expression pattern in which as many as 18% of the genes on the arrays were forced to be differentially expressed. The standard deviations of the differences between the true fold change and the observed fold change after normalization were computed for each of the modified genes.

In table 2, the first value in each value pair represents the percentage of standard deviations that were larger when the normalization technique listed in the leftmost column was compared against the normalization technique listed along the top row. For instance, the first value pair (0.94, 9.3) in cell (LR.1/LR.2, IDS.1/IDS.2) indicates that for the first set of data 94 percent of the LR.1 computed standard deviations were larger than the IDS.1 computed standard deviations, and that of those that were larger, the median LR.1/IDS.1 ratio of standard deviations was 9.3, i.e., half of the time, the LR.1 standard deviation is more than 9.3 times larger than the IDS.1 standard deviation.

Table 2, shows that the GCVSS technique makes the average difference intensities more consistent by destroying the very biological variation the technology aims to detect. That is, Table 2 shows how the GCVSS technique is too sensitive to the relatively small number of genes that change. On the other hand, the IDS/GCVSS technique continues to perform better than the other techniques, not only by minimizing the average difference intensity variation across replicates, but also by minimizing the deviation from the true fold change values in the simulated data.

As mentioned above in the introduction to this "Normalization" section, feature intensities may be used for normalization, too. Thus, finally, in another embodiment, normalization is performed on feature intensities rather than PM-MM differences. The method is the same as in the IDS/GCVSS method described above, except that the scatter plot of corresponding differences between the two arrays is replaced by the scatter plot of the corresponding feature intensity values between the two arrays.

Conclusion

Processes for feature extraction and normalization have been described. Because many important decisions as to whether a gene should be pursued as a candidate for a particular biological system under study are directly based on determined expression ratios as well as on the determined differential expression calls, processes that provide for more accurate estimates of these derived statistics can be valuable to users of oligonucleotide array technology.

It should be noted that the image processing techniques may have broader application beyond oligonucleotide arrays or even to microarrays, and claim to such broader application is not intended to be foreclosed by this disclosure.

WHAT IS CLAIMED IS:

1. A method of determining a characteristic intensity of a feature in image data generated by scanning a microarray probe, comprising:

identifying a set of pixels of the image data that nominally represent the feature, wherein the pixels each have an intensity value associated therewith;

for each of a plurality of subsets of the set of pixels, determining a variation statistic value that corresponds to a variation in the intensity values associated with the pixels of that subset; and

choosing one of the subsets of pixels based on the determined variation statistic values.

2. The method of claim 1, and further comprising:

determining the characteristic intensity of the feature based on the intensity values associated with the pixels of the chosen subset.

3. The method of claim 2, wherein the subsets of the set of pixels are determined in an iterative fashion starting from a "seed" set of pixels and forming subsequent subsets by adding additional pixels.

4. The method of claim 3, wherein each subsequent subset is chosen at each iteration from predetermined additional subsets to have the smallest determined variation statistic value of the predetermined additional subsets.

5. The method of claim 4, wherein the predetermined additional subsets all have substantially a predetermined shape.

6. A method of determining a characteristic value for a feature in image data generated by scanning a microarray probe, comprising:

identifying a set of pixels of the image data that nominally represent the feature, wherein the pixels each have a value associated therewith;

for each of a plurality of subsets of the set of pixels, determining a variation statistic value that corresponds to a variation in the values associated with the pixels of that subset;

choosing one of the subsets of pixels based on the determined variation statistic values.

7. The method of claim 6, and further comprising:

determining the characteristic value of the feature based on the intensity values associated with the pixels of the chosen subset.

8. The method of claim 7, wherein the subsets of the set of pixels are determined in an iterative fashion starting from a "seed" set of pixels and forming subsequent subsets by adding additional pixels.

9. The method of claim 8, wherein each subsequent subset is chosen at each iteration from predetermined additional subsets to have the smallest determined variation statistic value of the predetermined additional subsets.

10. The method of claim 9, wherein the predetermined additional subsets all have substantially a predetermined shape.

11. A method of relating a first expression array of probes to a second expression array of probes, comprising:

determining a subset of the probes for the arrays based on a comparison of the ordering of the subset of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes; and

determining a relationship of the second expression array to the first expression array based on the subset of probes of the second expression array to the corresponding probes of the first array.

12. The method of claim 11, wherein the step of determining a subset of probes includes:

selecting the determined subset of probes from a plurality of subsets of the probes.

13. The method of claim 12, wherein the selecting step comprises:

comparing the ordering of a first of the plurality of subsets of the probes of the second array, according to a particular characteristic of the probes, to the ordering of corresponding probes in the first array according to the particular characteristic of the probes;

if the comparison does not meet a particular criterion, repeating the comparing step with a second of the plurality of subsets of the probes of the second array, wherein the second subset is a subset of the first subset;

using the subset of the probes for which the comparison meets the particular criterion to determine the relationship of the second expression array to the first expression array.

14. The method of claim 13, wherein the particular criterion is a threshold.

15. The method of claim 14, wherein the threshold is not identical for all the probes.

16. The method of claim 13, wherein the step of using the probes to determine the relationship includes applying a nonlinear regression technique to the subset of the probes.

17. The method of claim 16, wherein the step of applying a nonlinear regression technique includes applying a generalized cross-validation step to the subset of the probes of the second array and the corresponding probes of the first array.

18. The method of claim 14, wherein:

the comparison D_i and threshold R_i are determined by the equations:

$$R_i = {[L(B_i + E_i) + H(2N - B_i - E_i)]}\big/{2N}$$

$$D_i = {2|B_i - E_i|}\big/{(B_i + E_i)}$$

where L and H are rank difference thresholds for the low and high ends of the range of characteristic values, B_i and E_i are the ranks for the i^{th} characteristic value of the baseline and experiment arrays, and N is the total number of characteristic values that were ordered in the current iteration of the method.

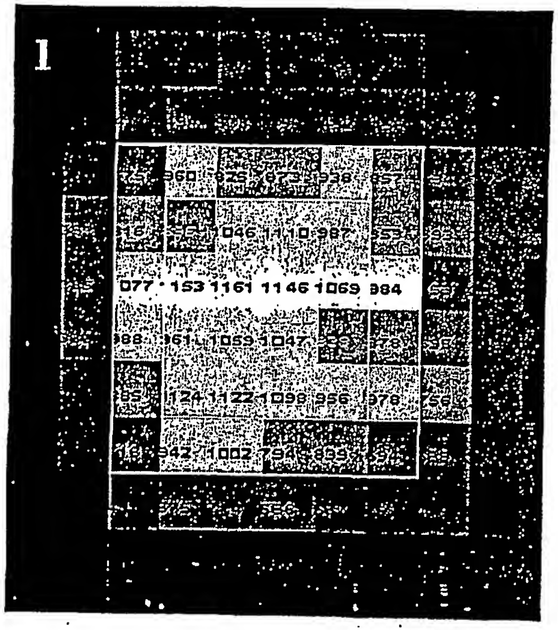

Fig. 1A

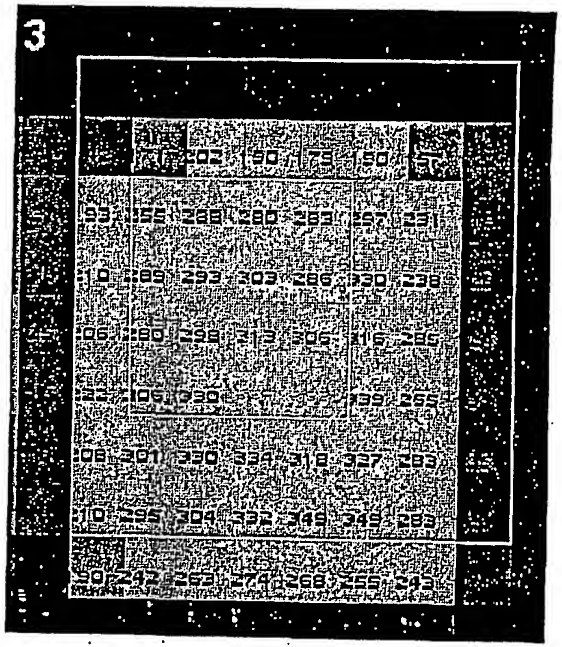

Fig. 1B

Prior Art

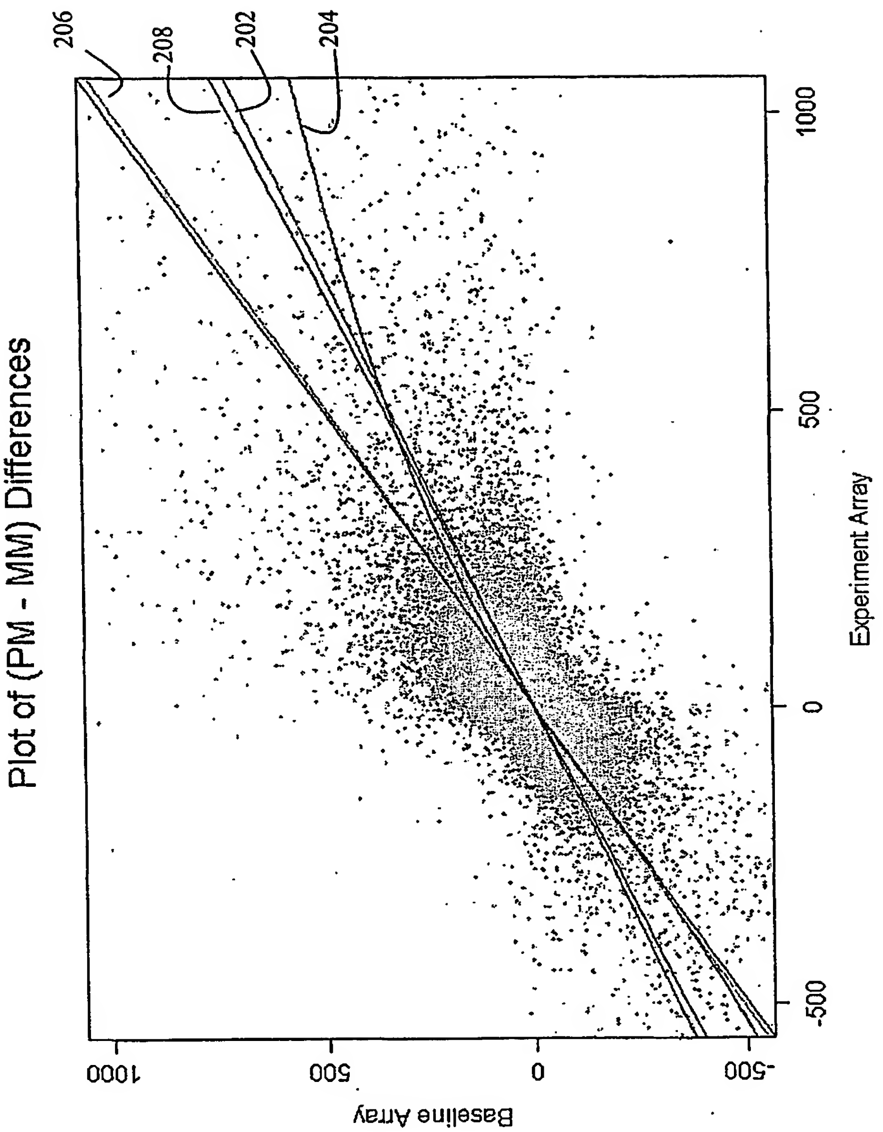
Plot of (PM - MM) Differences
206
208
202
204
Baseline Array
1000
500
0
-500
Experiment Array
-500
0
500
1000

Fig. 2

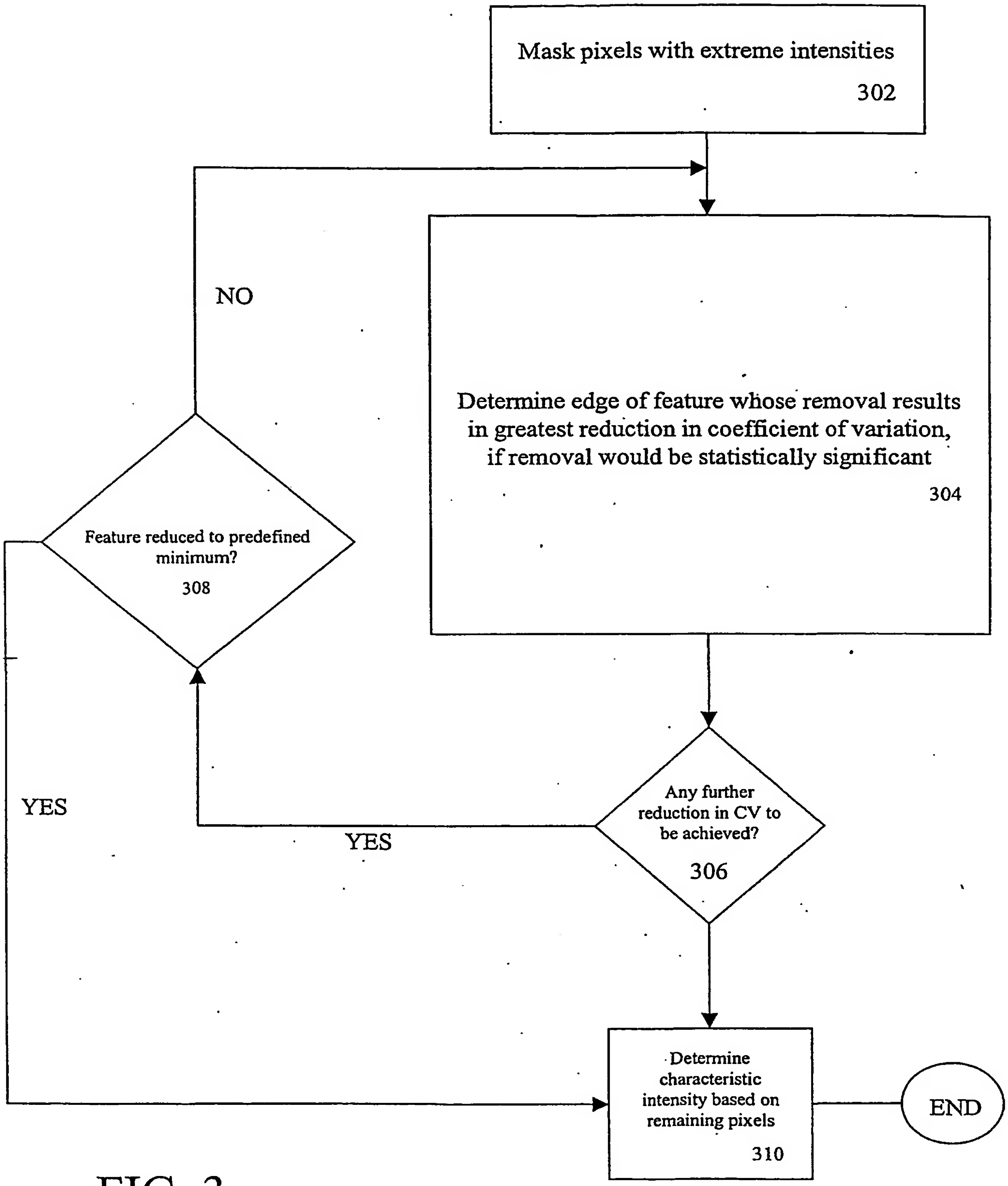
Mask pixels with extreme intensities
302
Determine edge of feature whose removal results in greatest reduction in coefficient of variation, if removal would be statistically significant
304
Any further reduction in CV to be achieved?
306
YES
Feature reduced to predefined minimum?
308
NO
YES
Determine characteristic intensity based on remaining pixels
310
END

FIG. 3

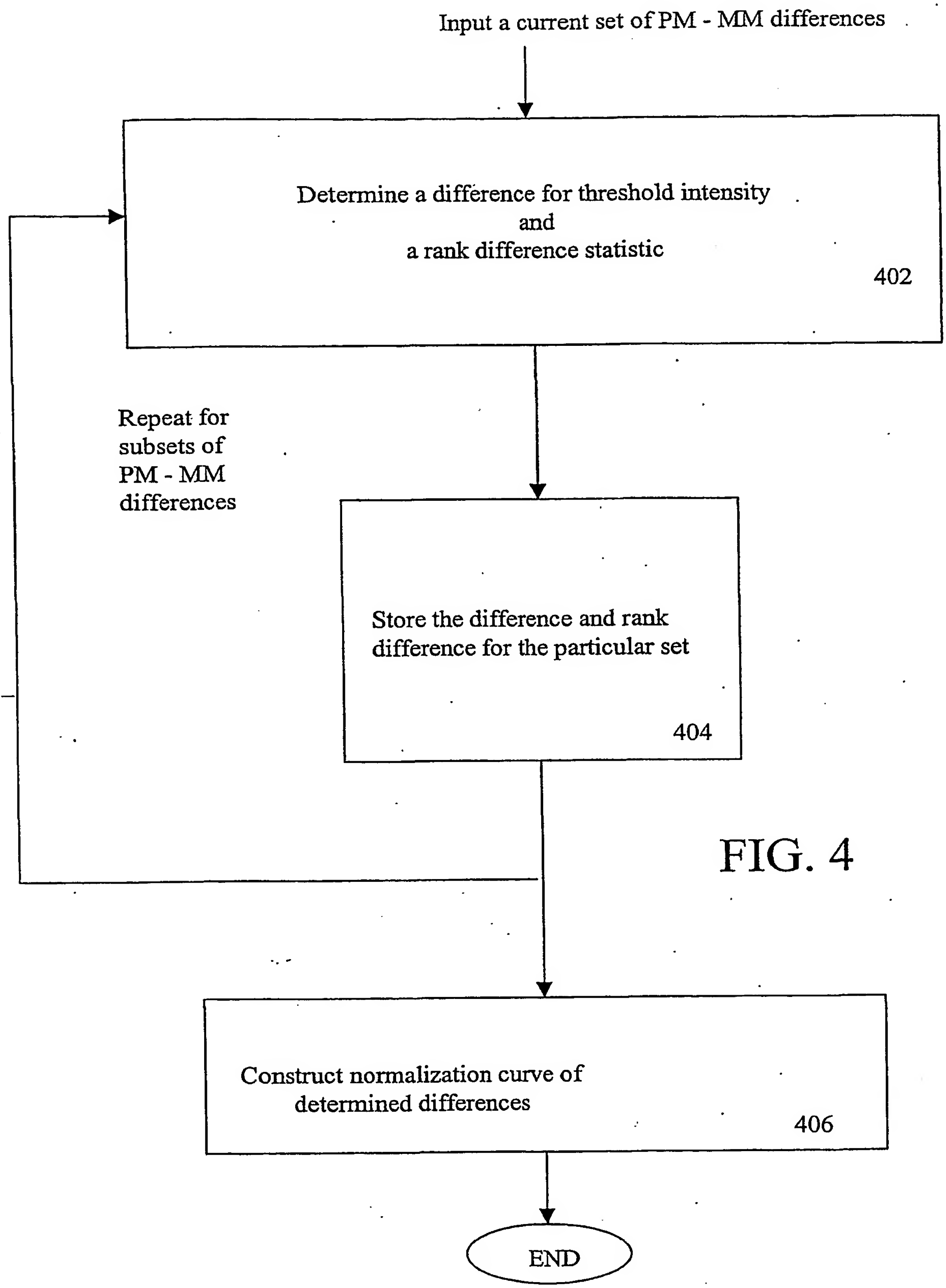
Input a current set of PM - MM differences
Determine a difference for threshold intensity and a rank difference statistic
402
Repeat for subsets of PM - MM differences
Store the difference and rank difference for the particular set
404
Construct normalization curve of determined differences
406
END

FIG. 4

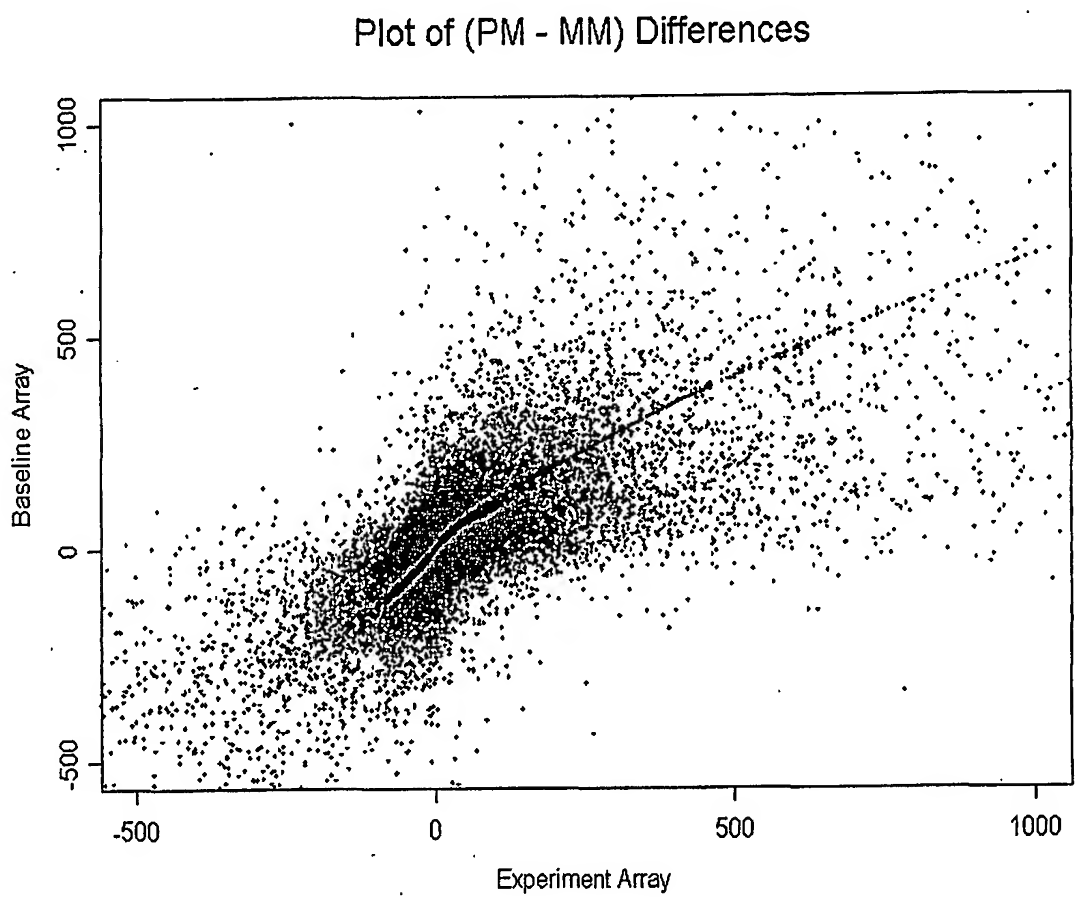
Plot of (PM - MM) Differences
Baseline Array
1000
500
0
-500
-500
0
500
1000
Experiment Array

Fig. 5